International Journal of Biochemistry and Cell Biology 109 (2019) 1–7

Contents lists available at ScienceDirect

International Journal of Biochemistry and Cell Biology

journal homepage: www.elsevier.com/locate/biocel

Hsa-miR-5195-3P induces downregulation of TGFβR1, TGFβR2, SMAD3 and SMAD4 supporting its tumor suppressive activity in HCT116 cells

Mahnaz Jahangiri Moez, Hassan Bjeije, Bahram M. Soltani*

Department of Genetics, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, Iran

ARTICLE INFO

Keywords:
Hsa-miR-5195-3p
TGFBR
TGFβ/SMAD signaling pathway

ABSTRACT

MicroRNAs are classified as small non-coding RNAs that regulate gene expression mainly through targeting the 3′UTR region of mRNAs. A great number of miRNAs play important role in the regulation of signaling pathways in normal and cancer cells. Here, we predicted *hsa-miR-5195-3p* (*miR-5195-3p*) as a potential regulator of TGFβ signaling and investigated its effect on *TGFB-R1*, *TGFB-R2*, *SMAD2, SMAD3* and *SMAD4* transcripts which are key players of TGFβ/SMAD signaling pathway. Overexpression of *miR-5195* in HCT116 cells resulted in a significant reduction of *TGFB-R1*, *SMAD2*, *SMAD3,* and *SMAD4* at the mRNA level which was confirmed using RT-qPCR. Consistently, western blot analysis confirmed that *miR-5195* overexpression in HCT116 cells resulted in downregulation of TGFBR1 at the protein level. Furthermore, dual luciferase analysis verified the direct interaction of *miR-5195* with *TGFB-R1* and *SMAD4* 3′UTR sequences in SW480 cells. Additionally, flow cytometry analysis confirmed that *miR-5195* overexpression significantly increased the sub-G1 and decreased the G-1 cell populations in both SW480 and HCT116 cell lines. Finally, *miR-5195* overexpression significantly downregulated *c-MYC* and *cyclin D1* but upregulated p21 genes. Overall, our results indicated that *miR-5195* modulates TGFβ signaling pathway and affects the cell cycle progression through targeting *TGFB-R1*, *TGFB-R2*, *SMAD2, SMAD3, SMAD4* transcripts.

1. Introduction

TGFβ/SMAD signaling pathway regulates many biological processes in different cell types including cell proliferation, differentiation and apoptosis (Tian et al., 2011; Jingushi et al., 2015). TGFβ superfamily ligands (TGFβ, bone morphogenic proteins (BMPs) and activin/inhibin) initiate Signal transduction through binding to the type 1 and type 2 receptor serine/threonine kinases on the cell surface. Type 2 receptor (TGFBR2) activates type 1 receptor (TGFBR1) via phosphorylation of the kinase domain of TGFBR1. TGFBR1 phosphorylates R-SMADs (SMAD2 and SMAD3) that will form a complex with the co-SMAD (SMAD4). Later on, this complex (SMAD2, SMAD3 and SMAD4) translocates into the nucleus as a transcriptional regulator (Miyazono, 2000; Shi and Massagué, 2003). Cooperation of various cofactors with the SMAD complex results in the expression of cell specific target genes (Massagué, Joan, 2012). Recent experimental studies indicated that TGF-β signaling exerts a tumor suppressive effects by promoting cell cycle inhibition and apoptosis during the early stages of carcinogenesis. On the contrary, TGF-β promotes tumor invasion and metastasis during the advanced stages of cancer (Siegel and Massagué, 2003; Lebrun, 2012). MicroRNAs are a class of small endogenous non-coding RNAs that generally bind to the 3′untranslated regions (3′-UTR) of target mRNAs and thus negatively regulate the expression of protein coding genes by inducing mRNA degradation or translational inhibition (Bartel, 2004; Ambros, 2004). Studies have indicated that miRNAs dysregulation play a crucial role in carcinogenesis by affecting the expression of tumor suppressive or oncogenic genes (Esteller, 2011). Several recent studies have introduced that miRNAs can directly modulate signal transduction through acting as a principal post-transcriptional regulators of gene expression. MiRNAs can cause different cellular responses through targeting signaling pathway components or regulators (Inui et al., 2010; Avraham and Yarden, 2012). Additionally, miRNAs have been reported to play an indispensable regulatory effect in TGFβ signaling (Hoballa et al., 2017; Jafarzadeh et al., 2016a). In the current study, we demonstrated that *hsa-miR-5195* exerts a negative regulatory effect on the gene expression of TGF-β/SMAD signaling components (Table 1).

* Corresponding author.
E-mail address: soltanib@modares.ac.ir (B.M. Soltani).

https://doi.org/10.1016/j.biocel.2019.01.001
Received 8 August 2018; Received in revised form 12 December 2018; Accepted 2 January 2019
Available online 16 January 2019

Table 1
Primer and oligo sequences that were used in the study.

Primer name	Primer sequences, 5' to 3'
miR-5195-3p	Forward: CAGATACAGCCCATCCTTCCAAG
	Reverse: CTGTCCGCTCTTACTCCCTC
TGFBR1-3'UTR	Forward: CTCGAGTTAATTCCTTTTTTGGCTAGGG
	Reverse: GCGGCCGCAACAAAAGCTTCATATCCTGGT
TGFBR2-3'UTR	Forward: AGTGGGTGACATAGAGCATTC
	Reverse: TACATGAGTACAGCAGAAGTGTT
SMAD3-3'UTR	Forward: CTCGAGCAGACCTCATGCCCAGCTCTCT
	Reverse: GCGGCCGCCCAGCAGCCTTGCCCTCATGTGTG
SMAD4 (PART1)-3'UTR	Forward: AGACCCACAACCTTTAGACTGAG
	Reverse: AGAAAAGGGAACAATGAGGCCA
SMAD4 (PART2)-3'UTR	Forward: AGGCAAGGAAACCAGATCC
	Reverse: CGACTATTTTTTAGCCACAGACTT
M13	Forward: GTAAAACGACGGCCAGTGAAT
	Reverse: CAGGAAACAGCTATGACCATG
pEGFPC1	Forward: AGTCCGCCCTGAGCAAAG
	Reverse: TACAAATGTGGTATGGCTGATTATG
Psi-check2	Forward: GAGGACGCTCCAGATGAAATG
	Reverse: CTCACACAAAAAACCAACACACAG
TGFBR1- real time	Forward: CATTTTTCCCAAGTGCCAGT
	Reverse: ACACCCCTAAGCATGTGGAG
TGFBR2- real time	Forward: TTTGGATGGTGGAAGGTCTC
	Reverse: GCAACAGCTATTGGGATGGT
SMAD2- real time	Forward: CGAGTGGGTAGTGTTCTCAGGG
	Reverse: TGG TCA GCT CCT TCT GGT GTG
SMAD3- real time	Forward: CCTGTGCTGGAACATCATCTCAG
	Reverse: CTTCCTAAGAGTCAAAGTCCCTGC
SMAD4- real time	Forward: AAGTAATGGCTCTGGGTTGGG
	Reverse: TCAAACAGCAGAACAAAGATAAGGAA
GAPDH	Forward: GCCACATCGCTCAGACAC
	Reverse: GGCAACAATATCCACTTTACCAG
miR-5195-3p- real time	Forward: ATCCAGTTCTCTGAGGGGG
U48	Forward: TGACCCCAGGTAACTCTGAGTGTGT
UNIVERSAL-outer	Reverse: GCGTCGACTAGTACAACTCAAG
Anchored OligodT	GCGTCGACTAGTACAACTCAAGGTTCTTCCAGTCACGACG(T)18V

2. Materials and methods

2.1. Bioinformatics

The 3′-UTR sequences of human TGFB-R1, TGFB-R2, SMAD2, SMAD3 and SMAD4 were retrieved using Entrez. Prediction of miRNA targets was conducted using Targetscan, microT-CDS and miRmap databases. In order to evaluate the MREs conservation in different organisms, blat search was performed using UCSC genome browser.

2.2. DNA constructs

Genomic DNA was extracted from the blood of a healthy individual according to the manufacturer's protocol (molecular cloning, Green and Sambrook) and used as a template for PCR. A 489 bp genomic DNA fragment containing the precursor of *hsa-miR-5195* was amplified using a pair of oligonucleotides and cloned into pEGFP-C1 expression vector (Clontech) downstream of the GFP sequence. *SMAD4*-3′UTR sequence containing MREs for *hsa-miR-5195* was divided into two fragments (2431bp and 2118bp in length) then were separately fused and cloned downstream of the luciferase reporter gene in psi-check2 vector. The PCR products were first cloned into TA vector (Fermentase) and then sub-cloned into psi-check2 Dual-Luciferase vector using NotI and XhoI restriction enzymes. All recombinant vectors were extracted using mini-prep kit (Qiagene Co.) and sequenced.

2.3. Cell culture

HCT116 and SW480 cell lines were cultured in RPMI- 1640 and HG-DMEM media (Invitrogen), respectively, supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma), 10% fetal bovine serum (FBS) (Invitrogen) and incubated at 37 °C with 5% CO2. These cell lines were obtained from Pasteur Institute/ Iran.

2.4. Transfection

For miRNA overexpression analysis, HCT116 and SW480 cell lines were seeded in 12 well plates (12 × 104 cells per well) and were allowed to adhere for 24 h. These cells were transiently transfected with the pEGFP-C1 expression vector containing *hsa-miR-5195* precursor using Lipofectamine 2000 (Invitrogen). Transfected cells were then cultured for 6 h and the culture media was replaced with a fresh media supplemented with 10% FBS. GFP expression was visually examined 24 h after transfection using fluorescent microscope (Nikon eclipses Te2000-s). The cells were harvested 36 h after transfection and RNA extraction was performed.

2.5. RNA extraction and quantitative RT-PCR

Total RNA was extracted from different cell lines using Trizol reagent according to the manufacturer's protocol (Invitrogen). The RNA quality and yield of the extracted RNA was analyzed using agarose gel electrophoresis and spectrophotometry, respectively. In order to avoid genomic DNA contamination, DNaseI treatment was performed prior to cDNA synthesis by the following method: DNAaseI treatment (Takara) at 37 °C for 30 min followed by inactivation at 72 °C with EDTA, then cDNAs were synthesized using Prime Script II reverse transcriptase (Takara). For miRNA detection, polyA tail was added to the 3′ end of RNAs before cDNA synthesis. Real-time PCR was performed using standard protocols of ABI PRISM7500 instrument (Applied Biosystems). Relative changes of gene expression were calculated by $2-\Delta\Delta Ct$ and $2-\Delta Ct$ methods and analyzed by GraphPad PRISM software. GAPDH and U48 sno-RNA expression levels were used as an internal control.

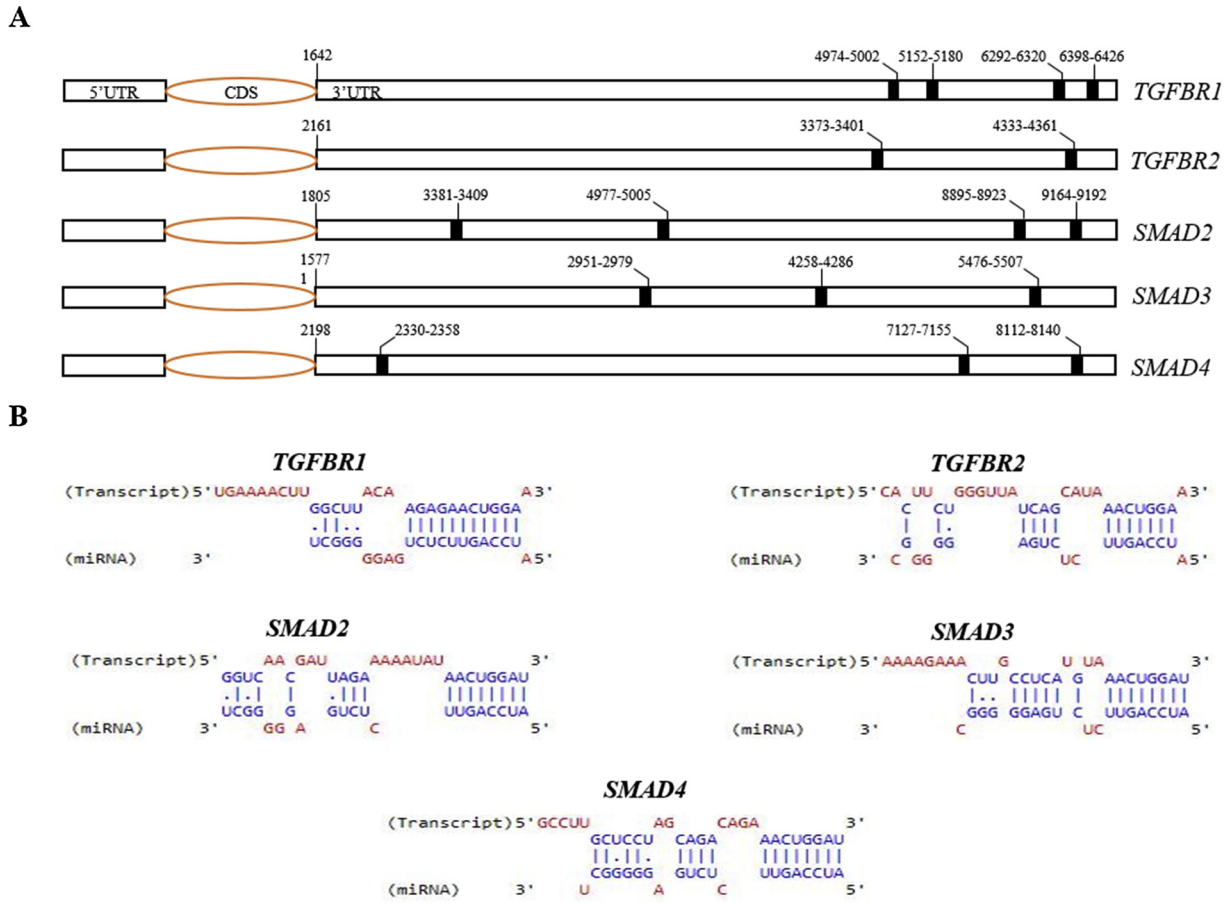

Fig. 1. Schematic representation of *miR-5195-3p* recognition sites (MREs) in the 3′UTR region of the target genes. A) Shows the Approximate position of miR-5195-3p specific MREs in the *TGFBR1, TGFBR2, SMAD2, SMAD3 and SMAD4 m*RNAs. The numbers over the MREs represent the distance from the transcription initiation site. B) Shows the pairing status of *miR-5195-3p* with one of the MRE sequences of the mentioned target genes.

2.6. Luciferase assay

For studying the direct interaction between *hsa-miR-5195-3p* and the 3′UTRs of *TGFB-R1*, *TGFB-R2*, *SMAD3* and *SMAD4*, SW480 cells were co-transfected with psi-check2 construct and pEGFP-C1 vector containing *hsa-miR-5195* precursor. Psi-check2 reporter construct contain Renilla luciferase gene upstream of 3′UTR of the target genes and an independent firefly luciferase gene which served as an internal control. 48 h after co-transfection of both vectors into SW480 cells, DualGlo luciferase assay was performed (Promega). The ratio of Renilla to Firefly luciferase was analyzed and normalized relative to the cells co-transfected with psi-check2 vector containing 3′UTR sequence of the target genes and pEGFP-C1 mock vector (empty vector).

2.7. Western blotting

Soluble proteins were extracted from HCT116 cells and subjected to 12% Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Sigma, USA), and then transferred to a polyvinylidene fluoride (PVDF, Thermo Scientific, USA) membrane. After that, membrane blocking was performed at RT for two hours using 5% milk (Sigma, USA) diluted in Tris-buffered saline containing 0.1% Tween (TBST, Bio basic, Canada). Later on, the membrane was incubated with the primary antibodies, TGFβ-R1 (1:500, Santa Cruz Biotechnology), cyclin D1 (1/1000, Santa Cruz Biotechnology) and β-actin (1:1000; Santa Cruz Biotechnology) overnight at 4 °C. Sheep anti-rabbit IgG-HRP (1/5000, Santa Cruz Biotechnology) (against TGFBR1 and cyclin D1) and goat anti-mouse IgG-HRP (1:3000; Santa Cruz Biotechnology) (against β-actin) secondary antibodies were incubated for 1 h at RT. Actin protein was used as a loading control. The bands were visualized using ECL reaction kit (Beyotime, China), recorded in Canon EOS 60D and quantitated using ImageJ software.

2.8. Cell cycle assay

SW480 and HCT116 cells were harvested 38 h after transfection with *hsa-miR-5195-3p*, then centrifuged at 1200 rpm for 5 min and washed twice in PBS. Subsequently, 1 ml of 70% cold ethanol was added and cells were fixed in this solution for at least 30 min. For each sample, 500 µl PI staining solution was added and incubated for 30 min at room temperature and then analyzed by FACS Calibur flow cytometer using the Cell Quest software (BD Biosciences).

3. Results

3.1. Bioinformatics study

DIANA, Mirmap, Targetscan, and miRWalk algorithms were used to search for the miRNAs that are potentially capable of targeting TGF-beta/SMAD signaling pathway components. Hsa-miR-5195-3p had several predicted recognition sites (MREs) within *TGFBR1*, *TGFBR2*, *SMAD3* and *SMAD4*-3′UTR regions. (Fig. 1). Compared to the flanking sequences of the 3′UTR region, these MREs were more conserved which will emphasize their functional importance.

3.2. Hsa-miR-5195-3p expression profile in different cell lines

RT-qPCR was performed to investigate the expression status of *miR-*

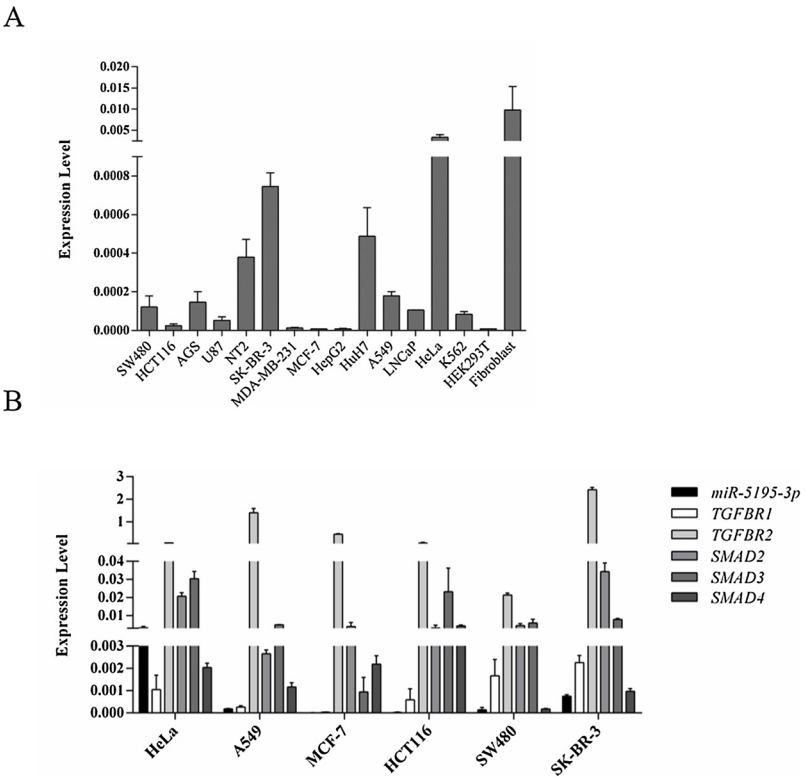

Fig. 2. Representation of *miR-5195-3p* and its predicted target genes expression level in different human cell lines detected by RT-qPCR. A) Shows the expression pattern of *miR-5195* in different cell lines. B) Shows the expression pattern of miR-5195 and TGF-B signaling genes in six different cell lines.

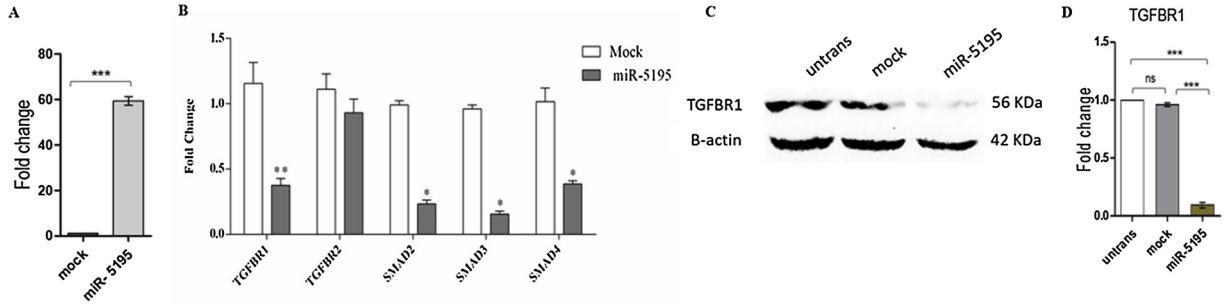

Fig. 3. The effect of *miR-5195* overexpression on TGFβ signaling pathway. A) Overexpression of *miR-5195-3p* precursor in HCT116 cells resulted in ˜60-fold increase in the expression level of the mature miRNA compared to the mock transfected cells. Expression data was generally normalized against *U48* as an internal control. B) RT-qPCR analysis of *TGFBR1, TGFBR2, SMAD2, SMAD3 and SMAD4* mRNA level following miR-5195 overexpression in HCT116 cell line. Results indicated that *TGFBR1, SMAD2, SMAD3 and SMAD4* endogenous expression level has been decreased. Expression data was normalized against *GAPDH* as an internal control. Asterisk mark (*) represent P-value < 0.01. C) Reduction of TGFBR1 protein level following *miR-5195* overexpression in HCT116 cells detected by Western blot. B-actin was used as an internal control. Results are the mean of three experiments. D) Protein band quantification of TGFBR1 was performed using image-J software in order to ensure the effect of *miR-5195* overexpression on TGFBR1 downregulation. Asterisk mark (*) represent P-value < 0.01.

5195-3p in different cell lines. The highest relative expression of *miR-5195-3p* was detected in fibroblast, SK-BR-3 and HeLa cells compared to other cell lines. However, its expression in HCT116 cells was relatively low (Fig. 2A). This low level *miR-5195-3p* expression makes HCT116 cells suitable for overexpression analysis. Then, the expression level of *miR-5195-3p*, along with *TGFBR1*, *TGFBR2*, *SMAD2*, *SMAD3*, and *SMAD4* predicted target genes was analyzed in Hela, A549, MCF-7, HCT116, SW480, and SK-BR-3 using RT-qPCR (Fig. 2B). Although *miR-5195-3p* was almost undetectable in MCF-7 (breast cancer cell line) and HCT116 (stage 4 colorectal cancer cell line) (Ahmed et al., 2013), the predicted target genes expression was relatively high in both of cell lines.

3.3. Hsa-miR-5195 overexpression promotes downregulation of TGFβ downstream genes

The effect of *miR-5195-3p* overexpression on the target genes expression level was investigated in HCT116 cell line that was transfected with pEGFP-C1 vector containing the precursor of this miRNA. Approximately, 60-fold change overexpression of *miR-5195-3p* was detected in the transfected cells compared to the mock control (Fig. 3A). *TGFBR1*, *SMAD2*, *SMAD3* and *SMAD4* mRNA levels were decreased following *miR-5195* overexpression in HCT116 cells however, this effect was not observed on *TGFBR2* after performing RT-qPCR (Fig. 3B). To confirm the effect of *miR-5195* on the TGFBR1 protein level, western blot analysis was performed on transfected HCT116 cells that were overexpressing this miRNA. Overexpression of *miR-5195* resulted in reduced TGFBR1 protein level in these cells, compared to the

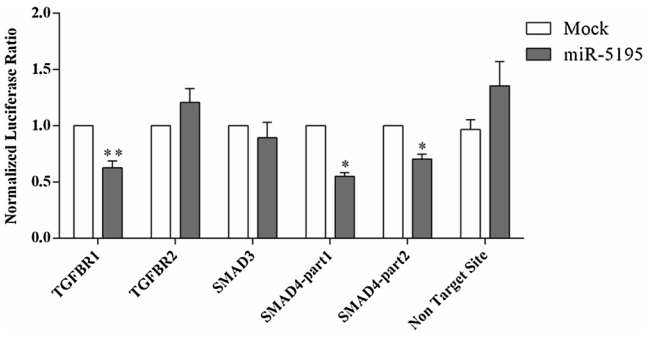

Fig. 4. Dual luciferase analysis to confirm the direct interaction *of miR-5195* with *TGFBR1, TGFBR2, SMAD3 and SMAD4* -3'UTR sequences. Reduction in the luciferase activity was detected in cells co-transfected with either TGFBR1 or SMAD4 3'UTR sequences and miR-5195 compared to the mock transfected cells. Asterisk mark (*) represent P-value < 0.01.

mock control (Fig. 3C). Protein band quantification of TGFBR1 was performed using image-J software in order to confirm the result's significance (Fig. 3D).

3.4. Direct interaction of miR-5195 with the candidate target genes 3'UTR sequences

Dual luciferase assay was performed in order to validate the direct interaction of *miR-5195* with 3'UTR sequences of *TGFBR1*, *TGFBR2, SMAD2*, *SMAD3* and *SMAD4* genes. This experiment confirmed the direct interaction of *miR-5195* with *TGFBR1* and *SMAD4* 3'UTR sequences in SW480 cells compared to the related controls. However, no significant change was detected in the luciferase activity of the cells co-transfected with *miR-5195 and TGFBR2* or *SMAD3* 3'UTRs (Fig. 4).

3.5. The effect of miR-5195 overexpression on cell cycle progression

In order to examine the effect of *miR-5195* overexpression on the cell cycle status, SW480 and HCT116 cells were transfected with pEGFP-C1::*miR-5195* or pEGFP-C1-mock vectors and PI flow cytometry assay was performed 36 h after transfection. Results indicated that the frequency of sub-G1 cell population in both cell lines has been significantly increased following *miR-5195* overexpression, compared to the mock and untransfected controls. Additionally, *miR-5195* overexpression significantly downregulated the frequency of G1 cell population in both cell lines, compared to the mock and untransfected controls (Fig. 5A/B).

3.6. The effect of miR-5195-3p overexpression on the cell cycle regulators

To confirm the effect of *miR-5195* overexpression on cell cycle progression at the molecular level, HCT116 cell line was transfected with pEGFP-C1::*miR-5195* or pEGFP-C1-mock vectors and the expression pattern of three cell cycle regulatory genes was studied. *Hsa-miR-5195* overexpression promoted downregulation of both cell cycle promoters *c-MYC* (at the mRNA level) (Fig. 6A) and cyclin D1 (at the mRNA and protein level) (Fig. 6A/B/C). However, it promoted upregulation of the cell cycle inhibitor $p21^{cip1/waf1}$ at the mRNA level (Fig. 6A). In order to confirm the cell cycle inhibitory effect of *miR-5195* at the protein level, western blot analysis was performed against cyclin D1 and the results showed that *miR-5195* overexpression significantly downregulated cyclin D1 protein level compared to the mock transfected cells (Fig. 6B). Protein band quantification of cyclin D1 was performed using image-J software in order to confirm the result's significance (Fig. 6C).

4. Discussion

TGF-β/SMAD signaling pathway effectively controls different biological processes and dysregulation of its canonical members occurs in different cancer types (Levy and Hill, 2006). This deregulation affects the tumor suppressive or oncogenic activity of TGF-β signaling and alters the characteristics of normal or cancer cell (Ikushima and Miyazono, 2010). MicroRNAs are a class of approximately 22 nucleotides short non-coding RNAs that act as a post-transcriptional regulators of gene expression. MiRNAs generally modulate signaling pathways that are involved in different biological processes (Chen, 2015; He and Gregory, 2004). As a potential master regulator of intracellular signaling pathway, miRNAs can negatively affect the activity of TGF-β/SMAD signaling through regulation of the expression of its components (Xia et al., 2014; Najafi et al., 2017). For instance, *hsa-miR-490-3p* represses cell migration in colorectal cancer by targeting TGFBR1 expression (Xu et al., 2015). *Hsa-miR-17-5p* targets TGFBR2 in gastric cancer and subsequently promotes proliferation and migration (Qu et al., 2016). *Hsa-miR-27b* inhibits TGFβ1-induced fibroblast activation by targeting TGFBR1 and SMAD2 (Zeng et al., 2017). *Hsa-miR-146a* modulates proliferation and apoptosis in gastric cancer by targeting SMAD4 (Xiao et al., 2012). We have demonstrated that *hsa-miR-497* and *hsa-miR-590* negatively regulate *SMAD3* expression elsewhere (Jafarzadeh et al., 2016a, b).

Here, we have used bioinformatic tools to predict and identify another miRNA that targets TGF-β/SMAD signaling components. *Hsa-miR-5195-3p* was suggested as a strong potential regulator of *TGFBR1*, *TGFBR2*, *SMAD2*, *SMAD3* and *SMAD4* transcripts through direct binding to their 3'UTR regions (Fig. 1). *Hsa-miR-5195-3p* has been related to leukemia (Schotte et al., 2011), associated with early stages of rectal cancer (Slattery et al., 2016), correlated with smoking induced colorectal cancer (Mullany et al., 2016) and gastric cancer stem cell (Liu et al., 2014) and has been downregulated in lipopolysaccharide (LPS)-induced periodontitis (Du et al., 2016). Recently, a new study confirmed the tumor suppressive activity of *miR-5195-3p* in non-small cell lung cancer through inhibition of cell proliferation, migration and invasion (Yang, 2018). Nevertheless, few studies have been reported concerning this miRNA to date. The endogenous expression level of *hsa-miR-5195-3p* was relatively low in most of the tested cell lines pointing to its potential tumor suppressive effect (Fig. 2). In order to examine the effect of *hsa-miR-5195-3p* overexpression on the target mRNA levels, its precursor was successfully overexpressed in HCT116 cell line (Fig. 3A). RT-qPCR showed that the endogenous transcript levels of *TGFBR1*, *SMAD2*, *SMAD3* and *SMAD4* were significantly decreased following *hsa-miR-5195-3p* overexpression (Fig. 3B). Consistently, TGFBR1 protein level was decreased following *hsa-miR-5195-3p* overexpression in HCT116 cells (Fig. 3 C/D). Then, dual luciferase assay supported that *hsa-miR-5195-3p* can effectively target *TGFBR1* and *SMAD4* 3'UTR sequences (Fig. 4).

Hsa-miR-5195-3p is reported to inhibit proliferation in bladder cancer cell lines and its overexpression decreased cyclinD1 protein level which is a regulatory molecule required for the cell cycle progression from G1 to S phase (Jiang et al., 2017). Consistently, flow cytometry analysis confirmed that the frequency of sub-G1 cell population was significantly increased following *miR-5195* overexpression in SW480 and HCT116 cell lines. Additionally, *miR-5195-3p* overexpression significantly downregulated the frequency of G1 cell population in both cell lines (Fig. 5). Furthermore, we confirmed the cell cycle inhibitory effect of *miR-5195* through measuring the expression level of three cell cycle regulatory genes. *Hsa-miR-5195* overexpression significantly downregulated the cell cycle promoters *c-MYC* proto-oncogene (at the mRNA level) (Fig. 6A) and *cyclin D1* (at the mRNA and protein level) (Fig. 6A/B/C), however, it increased the expression level of the cell cycle inhibitor $p21^{cip1/waf1}$ at the mRNA level (Fig. 6 A). One study has shown that TGFβ stimulates *cyclin D1* expression and promotes chondrocyte cell proliferation through activation of B-catenin and smad3 (Li

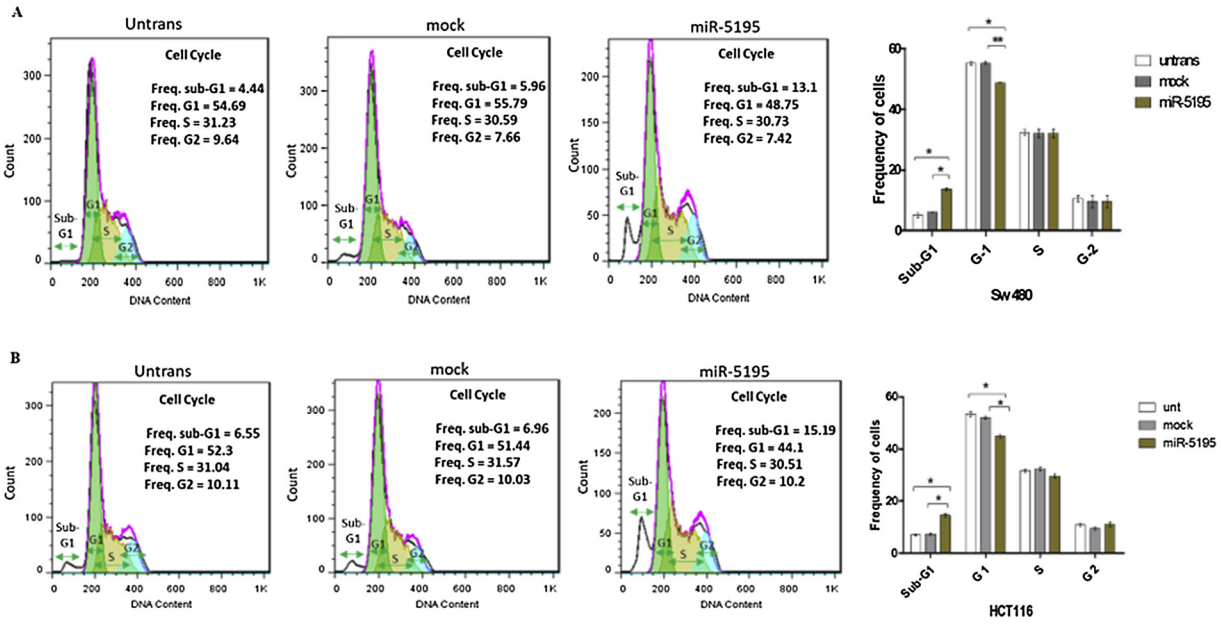

Fig. 5. Effect of *miR-5195-3p* overexpression on the cell cycle status. Following SW480 and HCT116 cells transfection with pEGFP-C1::*miR-5195* or pEGFP-C1-mock vectors for 36h, PI staining and flow cytometry analysis were performed. A) Cell cycle analysis of SW480 cell line showing a significant increase in the sub-G1 cell population and a significant decrease in the G1 cell population following *miR-5195* overexpression. B) Cell cycle analysis of HCT116 cell line also showing a significant increase in the sub-G1 cell population and a significant decrease in the G1 cell population following *miR-5195* overexpression. Results are the mean of duplicate experiments. Asterisk mark (**) represent p-value < 0.001.

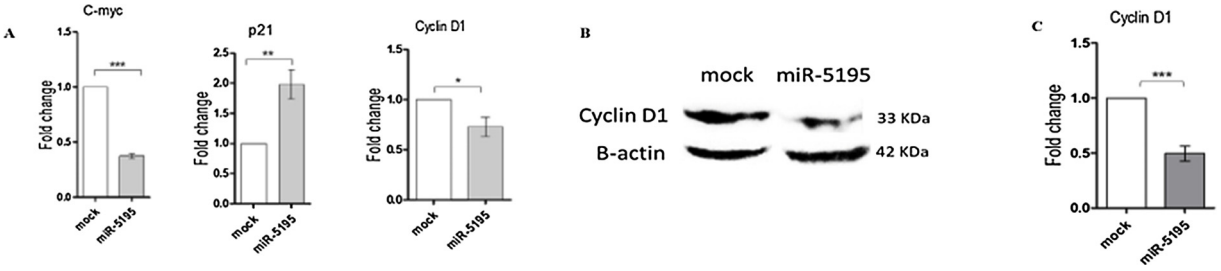

Fig. 6. The effect of *miR-5195* overexpression on the cell cycle regulatory genes. A) *Hsa-miR-5195* overexpression induced downregulation of both cell cycle promoters *c-MYC* and *cyclin D1*, however, it promoted upregulation of the cell cycle inhibitor $p21^{cip1/waf1}$ at the mRNA level. Asterisk mark (***) represent P-value < 0.0001. B) Western blot analysis against cyclin D1. Hsa-miR-5195 overexpression induced downregulation of cyclin D1 in comparison to the mock transfected cells. B-actin was used as an internal control. Results are the mean of three experiments. C) Protein band quantification of cyclin D1 was performed using image J software in order to ensure the effect of miR-5195 overexpression on cyclin D1 downregulation. Asterisk mark (***) represent P-value < 0.0001.

et al., 2006). On the other hand, several studies have shown TGFβ signaling as a promoter of tumor progression, invasion and metastasis in the advanced stages of cancer. Therefore, changes in the endogenous expression of TGFβ signaling components may influence the progression of the carcinogenic process in cancer cells (de Caestecker et al., 2000; Humbert et al., 2010). According to our results, the oncogenic activity of TGFβ signaling has been decreased following *miR-5195* overexpression in HCT116 cell which is known as a metastatic colon cancer cell line (Derry et al., 2013; Baker et al., 2011). This may justify the anti-proliferative effect of *miR-5195* overexpression in HCT116 cells (Fig. 5) which at least induced downregulation of TGFBR1 (Fig. 3B/C/D) and cyclin D1 (Fig. 6A/B/C). In conclusion, our results introduced *miR-5195-3p* as a tumor suppressor miRNA that negatively regulates TGFBR1 and other TGFβ signaling components and subsequently cell cycle progression. The current study introduces *miR-5195-3p* as a key therapeutic target for regulating TGFβ signaling pathway.

References

Ahmed, D., Eide, P.W., Eilertsen, I.A., Danielsen, S.A., Eknæs, M., Hektoen, M., Lind, G.E., Lothe, R.A., 2013. Epigenetic and genetic features of 24 colon cancer cell lines. Oncogenesis 1–8.

Ambros, Victor, 2004. The functions of animal microRNAs. Nature 431 (7006), 350–355.

Avraham, Roi, Yarden, Yosef, 2012. Regulation of Signalling by microRNAs. pp. 26–30.

Baker, Ann-Marie, Cox, Thomas R., Bird, Demelza, Lang, Georgina, Murray, Graeme I., Sun, Xiao-Feng, Southall, Stacey M., Wilson, Jon R., Erler, Janine T., 2011. The role of lysyl oxidase in SRC-dependent proliferation and metastasis of colorectal cancer. J. Natl. Cancer Inst. 103 (5), 407–424.

Bartel, David P., 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116 (2), 281–297.

Chen, Jiezhong, 2015. MicroRNAs, signaling pathways and diseases. Ann. Transl. Med. 3 (21).

de Caestecker, Mark P., Piek, Ester, Roberts, Anita B., 2000. Role of transforming growth factor-β signaling in cancer. J. Natl. Cancer Inst. 92 (17), 1388–1402.

Derry, Molly, Raina, Komal, Agarwal, Rajesh, Agarwal, Chapla, 2013. Differential effects of grape seed extract against human colorectal cancer cell lines: the intricate role of death receptors and mitochondria. Cancer Lett. 334 (1), 69–78.

Du, Anqing, Zhao, Sen, Wan, Ling Yun, Liu, Tian Tao, Peng, Zaoxia, Zhou, Zi Yu, Liao, Zhengyu, Fang, Huan, 2016. MicroRNA expression profile of human periodontal ligament cells under the influence of Porphyromonas gingivalis LPS. J. Cell. Mol. Med. 20 (7), 1329–1338.

Esteller, Manel, 2011. "Non-coding RNAs in human disease. Nat. Rev. Genet. 12 (12), 861–874.

He, Lin, Gregory, J., Hannon, 2004. MicroRNAs: small RNAs with a big role in gene regulation. Nat. Rev. Genet. 5 (7), 522–531.

Hoballa, Mohamad Hussein, Soltani, Bahram M., Mowla, Seyed Javad, Sheikhpour, Mojgan, Kay, Maryam, 2017. Identification of a novel intergenic miRNA located between the human DDC and COBL genes with a potential function in cell cycle arrest. Mol. Cell. Biochem. 1–8.

Humbert, L., Neel, J.C., Lebrun, J.J., 2010. Targeting TGF-beta signaling in human cancer

therapy. Trends Cell Mol. Biol. 5, 69–107.
Ikushima, Hiroaki, Miyazono, Kohei, 2010. TGFβ signalling: a complex web in cancer progression. Nat. Rev. Cancer 10 (6), 415–424.
Inui, Masafumi, Martello, Graziano, Piccolo, Stefano, 2010. MicroRNA control of signal transduction. Nat. Rev. Mol. Cell Biol. 11 (4), 252–263.
Jafarzadeh, Meisam, Soltani, Bahram M., Dokanehiifard, Sadat, Kay, Maryam, Aghdami, Nasser, Hosseinkhani, Saman, 2016a. Experimental evidences for hsa-miR-497-5p as a negative regulator of SMAD3 gene expression. Gene 586 (2), 216–221.
Jafarzadeh, Meisam, Bahram, M., Soltani, 2016b. Hsa-miR-590-5p interaction with SMAD3 transcript supports its regulatory effect on the TGFβ signaling pathway. Cell Journal. (Yakhteh) 18 (1), 7.
Jiang, Zhangjie, Zhang, Yida, Cao, Runfu, Li, Li, Zhong, Kezhao, Chen, Qingsheng, Xiao, Jianjun, 2017. MiR-5195-3p inhibits proliferation and invasion of human bladder cancer cells by directly targeting oncogene KLF5. Oncol. Res. Featur. Preclin. Clin. Cancer Ther. 25 (7), 1081–1087.
Jingushi, Kentaro, Ueda, Yuko, Kitae, Kaori, Hsae, Hiroaki, Egawa, Hiroshi, Ohshio, Ikumi, Kawakami, Ryoji, et al., 2015. miR-629 Targets TRIM33 to promote TGFβ/Smad signaling and metastatic phenotypes in ccRCC. Mol. Cancer Res. 13 (3), 565–574.
Lebrun, Jean-Jacques, 2012. The dual role of TGF in human cancer: from tumor suppression to cancer metastasis. ISRN Mol. Biol 2012.
Levy, Laurence, Hill, Caroline S., 2006. Alterations in components of the TGF-β superfamily signaling pathways in human cancer. Cytokine Growth Factor Rev. 17 (1), 41–58.
Li, Tian-Fang, Di Chen, Qiuqian Wu, Chen, Mo, Sheu, Tzong-jen, Schwarz, Edward M., Drissi, Hicham, Zuscik, Michael, O'Keefe2, Regis J., 2006. Transforming growth factor-β stimulates Cyclin D1 expression through activation of β-Catenin signaling in chondrocytes. J. Biol. Chem. 281 (30), 21296–21304.
Liu, Jianming, Ma, Lilin, Wang, Zhiwei, Wang, Lei, Liu, Chun, Chen, Ruixin, Zhang, Jianguo, 2014. MicroRNA expression profile of gastric cancer stem cells in the MKN-45 cancer cell line. Acta Biochim. Biophys. Sin. 46 (2), 92–99.
Massagué, Joan, 2012. TGFβ signalling in context. Nat. Rev. Mol. Cell Biol. 13 (10), 616–630.
Miyazono, Kohei, 2000. Positive and negative regulation of TGF-beta signaling. J. Cell. Sci. 113 (7), 1101–1109.
Mullany, Lila E., Herrick, Jennifer S., Wolff, Roger K., Stevens, John R., Martha, L., Slattery, 2016. Association of cigarette smoking and microRNA expression in rectal cancer: Insight into tumor phenotype. Cancer Epidemiol. 45, 98–107.
Najafi, Hadi, Soltani, Bahram M., Dokanehiifard, Sadat, Nasiri, Shirzad, Mowla, Seyed Javad, 2017. Alternative splicing of the OCC-1 gene generates three splice variants and a novel exonic microRNA, which regulate the Wnt signaling pathway. RNA 23 (1), 70–85.
Qu, Yanjun, Zhang, Haiyang, Duan, Jingjing, Liu, Rui, Deng, Ting, Bai, Ming, Huang, Dingzhi, et al., 2016. MiR-17-5p regulates cell proliferation and migration by targeting transforming growth factor-β receptor 2 in gastric cancer. Oncotarget 7 (22), 33286.
Schotte, Diana, Akbari Moqadam, F., Lange-Turenhout, E.A.M., Chen, Connie, Van IJcken, W.F.J., Pieters, Rob, Den Boer, M.L., 2011. Discovery of new microRNAs by small RNAome deep sequencing in childhood acute lymphoblastic leukemia. Leukemia 25 (9), 1389–1399.
Shi, Yigong, Massagué, Joan, 2003. Mechanisms of TGF-β signaling from cell membrane to the nucleus. Cell 113 (6), 685–700.
Siegel, Peter M., Massagué, Joan, 2003. Cytostatic and apoptotic actions of TGF-β in homeostasis and cancer. Nat. Rev. Cancer 3 (11), 807–820.
Slattery, Martha L., Herrick, Jennifer S., Pellatt, Daniel F., Mullany, Lila E., Stevens, John R., Wolff, Erica, Hoffman, Michael D., Wolff, Roger K., Samowitz, Wade, 2016. Site-specific associations between miRNA expression and survival in colorectal cancer cases. Oncotarget 7 (37), 60193.
Tian, Maozhen, Neil, Jason R., William, P., 2011. Schiemann. "Transforming growth factor-β and the hallmarks of cancer. Cell. Signal. 23 (6), 951–962.
Xia, Qing, Li, Chengjiang, Bian, Pingda, Wang, Jue, Dong, Shuai, 2014. Targeting SMAD3 for inhibiting prostate cancer metastasis. Tumor Biol. 35 (9), 8537–8541.
Xiao, Bin, Zhu, En-Dong, Li, Na, Lu, Dong-Shui, Li, Wei, Li, Bo-Sheng, Zhao, Yong-Liang, et al., 2012. Increased miR-146a in gastric cancer directly targets SMAD4 and is involved in modulating cell proliferation and apoptosis. Oncol. Rep. 27 (2), 559–566.
Xu, Xuehu, Chen, Rong, Li, Zhifa, Huang, Nanqi, Wu, Xiaobing, Li, Shuling, Li, Yong, Shangbiao, Wu., 2015. MicroRNA-490-3p inhibits colorectal cancer metastasis by targeting TGFBR1. BMC Cancer 15 (1), 1023.
Yang, Quanfu, 2018. MicroRNA-5195-3p Plays a Suppressive Role in Cell Proliferation, Migration and Invasion by Targeting MYO6 in Human Non-small Cell Lung Cancer" Bioscience. Biotechnology, and Biochemstry, pp. 1–9.
Zeng, Xiangming, Huang, Chaoqun, Senavirathna, Lakmini, Wang, Pengcheng, Liu, Lin, 2017. miR-27b inhibits fibroblast activation via targeting TGFβ signaling pathway. BMC Cell Biol. 18 (1), 9.